INHIBITION OF FUNGAL GROWTH BY BACTERIA DURING CELLULOSE-DECOMPOSITION[1]

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Abstract

The cellulose-decomposing flora developing from soil crumbs on filter paper placed upon an agar medium was studied. Under these conditions fungal development was significantly inhibited by bacterial growth. When cellophane was substituted for filter paper, no inhibition of fungal growth was observed. A pure culture of *Cellvibrio* inhibited the vegetative growth of *Stachybotrys* when inoculated simultaneously on media containing carboxymethyl-cellulose, filter paper, or glucose up to 0.1%. This inhibition was shown to be caused by competition for available carbohydrates.

Introduction

Although the ability to decompose cellulose is widespread among many groups of soil bacteria and fungi, a rather specific cellulose-decomposing flora has been described under specified conditions by different workers. For example, *Cellvibrio* and *Cytophaga* were shown by Winogradsky (14) to predominate in the microflora developing around soil crumbs placed on silica-gel plates covered with filter paper. Stanier obtained inhibition of fungal growth and development of cellulolytic bacteria while using water-saturated filter paper placed upon mineral agar (11). On the other hand, Tribe's investigations, in which glass slides covered with cellophane or filter paper were buried in the soil, showed that cellulolytic fungi were the first organisms to attack cellulose in the soil (13).

The contradictory findings of these investigators may be explained by differences in physical conditions (pH, oxygen tension, temperature, humidity) or differences in the type of cellulose (3). The inhibition of fungal growth on agar plates, however, may also be the result of the competition for nutrients between bacteria and fungi or of the production of antibiotic or toxic substances.

The present paper describes investigations on the effect of some of these factors on the selection of the cellulose-decomposing flora on agar and liquid media.

Materials and Methods

Soils.—Unless otherwise stated samples of brown-red sandy soil (pH 7.2) from the garden of the Faculty of Agriculture, Rehovot, Israel, were used. In addition, samples of neutral and slightly alkaline soils taken from various locations in Israel were tested as indicated in the text.

Fungal and bacterial strains.—A cellulolytic strain of the mold *Stachybotrys* and an unidentified species of the bacterial genus *Cellvibrio* were isolated from soil and used as test organisms. Spore suspensions of *Stachybotrys* were

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prepared from a 7-day-old culture on yeast dextrose agar. The agar surface was washed with distilled water, and the spores were collected and washed 3 times with 20 ml of distilled water by centrifugation. The population densities of the spore suspensions were determined with a haemocytometer counting chamber. Bacterial suspensions were prepared from 2-day-old cultures grown on agar containing 1% of lactose at 30° C. The suspensions were washed with saline by centrifugation and resuspended to give an optical density of 0.4 at 550 mμ when measured in a Coleman Junior spectrophotometer, using 19×150 mm test tubes.

Basic medium for testing cellulose-degradation and growth relations between fungi and bacteria.—The medium used contained 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgCl_2.6\ H_2O$, 0.01% Bacto yeast extract (Difco), in 1 liter of distilled water, pH 7.0. For a solid medium, 1.5% Bacto agar (Difco) was added. The carbon sources added to this medium were: Whatman filter paper No. 1; cellulose film, Grade P.T. 300;* carboxymethylcellulose (ICI), specific viscosity of 5.6 at a concentration of 1%, or glucose in various concentrations. The cellophane was treated overnight with 0.1 *M* versene at pH 8.0, and then washed thoroughly with distilled water and boiled for 30 minutes.

Determination of fungal growth.—The fungal mycelium was collected from the liquid medium by centrifugation, washed twice with distilled water, and dried in an oven at 100° C to a constant weight. Growth was expressed as mg dry weight/100 ml medium.

Bacterial growth in liquid medium.—This was determined by measuring optical density of bacterial suspensions in a Coleman Junior spectrophotometer at 550 mμ, using 19×150 mm test tubes.

Reducing sugar.—This was determined by the method of Somogyi (10) and Nelson (7), values being expressed as mg glucose/ml.

Estimation of cellulose-decomposition on agar media.—Strips of filter paper or cellophane, 1 × 8 cm, were placed on agar plates and two soil crumbs (average diameter 2 mm) were placed at equal distances on each strip. Cellulose decomposition was estimated by lifting the strips carefully by means of sterile forceps and determining the time required for decomposition to proceed to such an extent that the two portions of the strip on either side of the soil crumb came apart. When pure cultures were tested, suspensions of the organisms were placed on one location in the middle of the strip.

Experimental and Results

A total of 72 strips of filter paper (width 1 cm) and cellophane, respectively, were placed on agar plates, half of which contained 100 μg/ml chloramphenicol (Lepetit). Two soil crumbs were placed on each of the strips (288 soil crumbs in all). The plates were incubated for 11 days at 30° C and tested for cellulose degradation and microbial growth. The results are summarized in Table I. On filter paper in the absence of chloramphenicol growth was predominantly bacterial. On medium with cellophane both with and without the antibiotic, fungi were the dominant organisms. This experiment was repeated

*Kindly supplied by the British Cellophane Co., Ltd., Bridgwater, Somerset, England.

TABLE I

Cellulose degradation and microbial growth around soil crumbs on agar medium

Substrate	Chloramphenicol (100 μg/ml)	Cellulose degradation/growth ratio*	
		Fungi	Bacteria
Filter paper	−	3/3	69/72
Filter paper	+	70/72	0/0
Cellophane	−	66/72	0/4
Cellophane	+	72/72	0/4

* $\frac{\text{Numerator}}{\text{Denominator}} = \frac{\text{No. of soil crumbs around which cellulolytic activity was observed}}{\text{No. of soil crumbs with microbial growth}}$.

TABLE II

The effect of chloramphenicol on the degradation of filter paper and microbial growth around soil crumbs on agar medium

Incubation time: 11 days at 30° C

Soil type	Chloramphenicol (100 μg/ml)	Cellulose degradation/growth ratio*	
		Fungi	Bacteria
Loamy sand (20 years of citrus growth)	−	2/2	20/20
	+	20/20	0/0
Loamy sand (9 years of citrus growth)	−	0/0	20/20
	+	20/20	0/0
Soil from a ploughed unirrigated field plot	−	12/12	1/20
	+	20/20	0/0
Alluvial soil, 4 years' irrigation (alfalfa)	−	0/2	17/20
	+	19/20	0/0
Alluvial plain, unirrigated	−	8/12	2/20
	+	6/20	0/0
A virgin sand dune	−	17/18	0/0
	+	18/20	0/0

*As in Table I.

with crumbs of soil samples collected from various locations in Israel. From soil crumbs taken from cultivated and well-irrigated soils and placed on filter paper with chloramphenicol, mainly bacteria developed. Soil crumbs from an uncultivated sand dune showed fungal growth both on filter paper with and without the antibiotic (see Table II).

Most of the cellulolytic bacteria which grew on the filter paper strips belonged to the genus *Cellvibrio*, while some decomposed cellulose in a manner typical of myxobacteria. Some of the cellulolytic fungi were identified as *Stachobotrys*, *Aspergillus*, *Fusarium*, *Curvularia*, *Cephalosporium*, and *Chaetomium*.

The results described above could be explained by the antagonistic action of cellulose-decomposing bacteria towards cellulolytic soil fungi when filter paper was used as the sole carbon source. To test this hypothesis, experiments with pure cultures were carried out.

Pieces of filter paper 1.5 × 1.5 cm were placed upon 12 ml agar in a Petri dish (5 squares on each plate). Twenty pieces were inoculated by means of a micropipette with spores of *Stachybotrys* (2.5×10^3 spores in 0.01 ml on each piece) and 20 with both *Stachybotrys* and *Cellvibrio*. The plates were incubated at 30° C and observed by direct microscopy for fungal growth and sporulation. All pieces inoculated both with *Stachybotrys* and *Cellvibrio* showed a very limited mycelial growth and no sporulation, whereas pieces inoculated with *Stachybotrys* alone showed mycelial growth after 24 hours and sporulation after 48–72 hours. When the experiment was repeated with cellophane instead of filter paper, the growth of *Stachybotrys* was not inhibited by *Cellvibrio*. This may result from the limited growth of *Cellvibrio* on cellophane, while filter paper placed on the same solid agar medium was readily decomposed by *Cellvibrio*.

Influence of Cellvibrio on the Germination of Spores of Stachybotrys

Since Dobbs and Hinson (1) found that germination of fungal spores was inhibited by natural soil, it was considered that the antagonistic effect of *Cellvibrio* on *Stachybotrys* might also result from an inhibition of spore germination. Petri dishes with 10 ml of liquid medium containing 1% carboxymethylcellulose as carbon source were inoculated with 1 ml of a solution containing 0.5×10^4 *Stachybotrys* spores and 0.1 ml of a suspension of *Cellvibrio* (optical density = 0.4 at 550 mμ) and incubated at 30° C. Inhibition of mycelial growth was observed when compared to controls without bacteria. However, 96% of the spores germinated normally within 8 hours in cultures both with and without *Cellvibrio*. Similar results were obtained with filter paper placed upon agar and inoculated in the same way.

The Fungistatic Effect of Cellvibrio on Stachybotrys

Pieces of sterile filter paper, 1.5 × 1.5 cm, placed on agar medium were inoculated with 0.01 ml of *Stachybotrys* spores. Some of the pieces were simultaneously inoculated with 0.01 ml of *Cellvibrio* culture. The plates were incubated at 30° C. Pieces bearing the mixed populations, which did not show any visible fungal growth, were transferred daily to agar plates containing 100 μg/ml chloramphenicol and incubated for 3 more days. The results are summarized in Table III. The survival of the germinating spores in the

TABLE III

Fungistatic effect of *Cellvibrio* upon *Stachybotrys* on filter paper

Incubation time before transfer to medium containing chloramphenicol (hours)	Number of pieces of filter paper placed on agar	Number of *Stachybotrys* colonies	Colonies*/pieces of filter paper
0	50	248	4.96
8	57	283	4.96
24	52	250	4.80
48	50	219	4.38
96	38	155	4.08
Stachybotrys alone	13	64	4.92

*Average number of fungal colonies per single piece of filter paper.

presence of *Cellvibrio* during 4 days indicated clearly that the effect of *Cellvibrio* upon the vegetative growth of *Stachybotrys* is mainly fungistatic. Statistical analysis of the data showed that there was no significant decrease in number of colonies per piece.

The Effect of Glucose on the Antagonistic Action

Duplicates of Roux bottles containing 100 ml of liquid medium, prepared with various concentrations of glucose, were inoculated either with *Stachybotrys* spores (2.5×10^3/ml final concentration) or cells of *Cellvibrio* (0.5 ml, optical density 0.4 at 550 mμ) or both. After an incubation period of 5 days at 30° C the fungal mats were collected by centrifugation, washed twice with distilled water, dried overnight at 100° C, and weighed. Bacterial growth was determined by measuring the optical density. The results are summarized in Fig. 1. It may be seen that fungal growth was markedly inhibited by *Cellvibrio* only at the low concentrations of glucose, the levels at which bacterial growth responded most noticeably to additional carbohydrate. At higher concentrations of glucose, bacterial response was small and fungal inhibition decreased sharply, until at 2.0 mg/ml little antagonistic effect could be observed.

The quantities of reducing sugar, which remained in the medium after the growth of *Cellvibrio* alone were also determined. It was found that in media containing up to 1.0 mg/ml of glucose (initial concentration), no reducing sugar was left by the bacteria after 5 days of incubation; at higher initial concentrations of glucose, reducing sugar was still present after 5 days

The Effect of Carboxymethylcellulose on the Antagonistic Action of Cellvibrio

When the experiment described above was carried out with carboxymethylcellulose (1%) as the sole carbon source, 12.6 mg mycelium dry weight/100

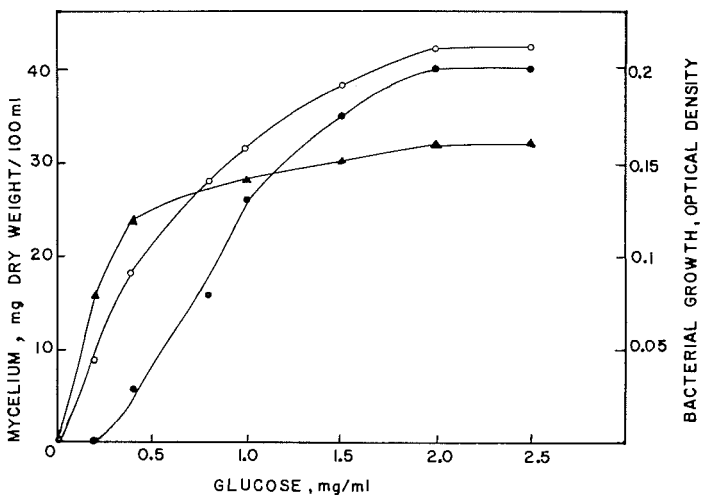

FIG. 1. Effect of various concentrations of glucose on the antagonistic activity of *Cellvibrio* on *Stachybotrys*. ○ *Stachybotrys*. ▲ *Cellvibrio*. ● *Stachybotrys* in the presence of *Cellvibrio*.

ml medium were found when *Stachybotrys* was grown alone, while in the presence of *Cellvibrio* only 3.1 mg/100 ml were found. No reducing sugar was detected.

Discussion

Fungal development from soil crumbs was significantly inhibited when filter paper was used as the sole carbon source. When *Stachybotrys* and *Cellvibrio* strains isolated from soil were inoculated together on filter paper, only *Cellvibrio* developed. As antagonistic effects occurring in nature between microorganisms may be caused by the excretion by one organism of substances toxic to another, such a possibility was considered. There is good evidence, however, that no such substance was formed since growth of *Stachybotrys* could be initiated even after the fungus had been exposed to *Cellvibrio* for some days in the same medium. In addition, no inhibition of the growth of *Stachybotrys* by *Cellvibrio* in a liquid medium was observed when the glucose concentration was sufficient to support growth of both organisms. Apparently, the suppression results from a competition for degradation products of cellulose rather than for cellulose itself, since this competition could also be demonstrated when glucose was used as sole carbon source. The finding that no reducing sugar could be detected whenever antagonism was evident supported the conclusion that a competition for available carbohydrates rather than for cellulose was responsible for the inhibition.

In addition to the production of antibiotics and toxic substances, competition between microorganisms for nutrients is probably also playing a role in microbial interactions in the soil (5, 12). Moreover, available nutrients in the soil are at a much lower level than in ordinary culture media used for antibiotic production (2). Competition for nutrients, however, is not the sole mechanism responsible for inhibition of fungal growth, other mechanisms such as inhibition of germination of fungal spores (1) and lysis of fungal mycelium (4) by unknown substances must be taken into account. Competition for nutrients, however, may be a secondary cause for the lysis of fungal mycelium in soil, as autolysis of mycelium is well known to be much stronger in poor than in rich media (15), i.e., in the absence of available nutrients.

An antagonistic effect of *Bacillus macerans* upon various soil fungi was also observed by Park (8), who studied the behavior of soil fungi in the presence of bacterial antagonists. However, no attempt was made to find out the nature of this antagonism.

The type of cellulose used as substrate seems also to be of importance since inhibition of fungal growth was observed only when filter paper or carboxymethylcellulose was used as carbon source. When cellophane was used as the substrate, fungi grew normally on agar even in the presence of bacteria. This can be explained by the limited ability of the bacteria tested to decompose cellophane on an agar medium. Native and regenerated cellulose differ in a number of chemical and physical properties such as associated substances, crystal structure, properties of the crystalline material, degree of swelling, etc. (6). These differences possibly account for the observed differences in microbial growth when either cellophane or filter paper was

used as a sole carbon source. In contrast to fungi, bacteria lack the ability to penetrate actively into solid substrates (9). In the case of cellophane, fungi grow into this substrate whereas bacteria remain confined to the surface. On filter paper, on the other hand, the competition is apparently on the surface of the microscopic fibrils which are covered with a film of water, and the bacteria apparently dominate in this water film. It may be suggested that bacteria can (by using this water film as a means of transportation), reach the fungal mycelium whenever it grows and compete with it for degradation products of the cellulose.

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